

SYNTHESIS OF MYCORRHIZAE ON EXPLANTS OF THE ROOT HYPOCOTYL OF *PINUS SYLVESTRIS* L.¹

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Abstract

A method of growing excised root cultures of leguminous plants was modified for the culture of excised roots of *Pinus sylvestris* L. The inclusion of a portion of hypocotyl on the explants from the seedlings was found essential to obtain sustained growth of the root. With such explants, typical ectotrophic mycorrhizae were obtained upon inoculation with *Amanita rubescens* (Pers. ex Fr.) Quel.

Introduction

Modifying White's technique, Slankis (1947) successfully cultivated isolated roots of *Pinus sylvestris* L. Upon addition of a mycorrhizal fungus to such a root culture, a certain stimulation could be observed, both of the fungus (Melin 1963) and of the root (Slankis 1948), but on no roots did typical mycorrhizae form.

Similarly, Lewis and McCoy (1933), by the use of the classical procedure (Robbins 1922), failed to obtain nodules on excised roots of legumes; but later Raggio *et al.* (1956-1957) devised a method by which effective nodules were obtained on excised root culture. Their technique consisted essentially in dividing the culture medium into two parts, one containing the organic substances, the other, the mineral components. The organic constituents are solidified with agar and fed to the excised root by its upper part, while the mineral solution is available only to the lower, normally subterranean, part of the root.

The present paper deals with the modification of this technique to permit the formation of ectotrophic mycorrhizae on excised roots of *P. sylvestris*.

Materials and Methods

Seeds of *P. sylvestris* were sterilized by immersion in 30% H₂O₂ for 5 to 10 seconds. The peroxide had been previously equilibrated with room temperature, and a pinch of detergent added to act as a wetting agent. The excess peroxide was removed by placing the seeds in contact with a paper towel moistened with the sterilizing agent. Thereafter the seeds were placed, five at a time, into previously autoclaved 250-ml Erlenmeyer flasks containing 100 cm³ of vermiculite and 50 ml of the following solution adapted from that of Shive (Bonner and Galston 1959).

Ca(NO ₃) ₂ ·4H ₂ O	106.0 mg
KH ₂ PO ₄	31.0 "
MgSO ₄ ·7H ₂ O	55.0 "
(NH ₄) ₂ SO ₄	9.0 "
Ferric citrate - citric acid (1.0% solution)	1.0 ml
Trace elements solution (as below)	1.0 "
Distilled water to	1000.0 "

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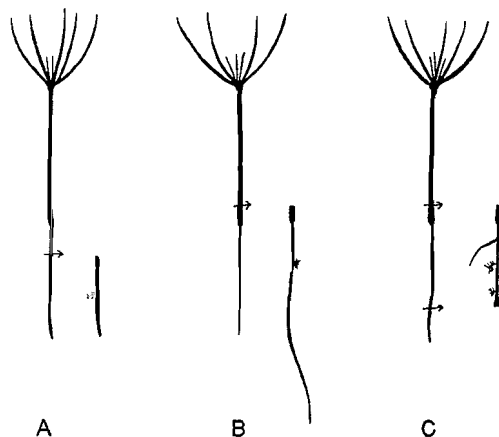


FIG. 1. Methods of excising the roots. (A) Excision of only the root gave no growth. (B) Excision of the root with some hypocotyl attached gave normal growth. (C) Excision of the root with some hypocotyl attached after removal of the apical meristem gave the development of numerous short laterals.

Trace elements solution (Johnson *et al.* 1957)

KCl	3.728 g
H ₃ BO ₃	1.546 "
MnSO ₄ ·H ₂ O	0.845 "
ZnSO ₄ ·7H ₂ O	0.575 "
CuSO ₄ ·5H ₂ O	0.125 "
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.018 "
Distilled water to	1000.0 ml

The seeded flasks were exposed to daylight and artificial light (minimum, 1400 ft-c) for 16 hours a day. After a predetermined time (15 days for growth studies, 30 days for studies on mycorrhiza formation), the seedlings were removed from the flasks and the roots were excised. Generally, no lateral rootlets were present at the time of excision.

In the first attempts to grow the isolated roots, the excision was made 25 mm behind the root apex (Fig. 1a). During this work, however, it was found essential to keep a part of the hypocotyl on the explant. Thereafter, the excision was made 5 mm above the junction between hypocotyl and root. This gave fast-growing long roots with few short laterals (Figs. 1b and 4). Furthermore, during attempts to increase the production of the short laterals (where the

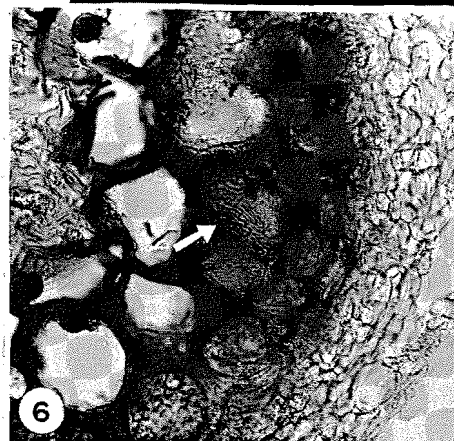
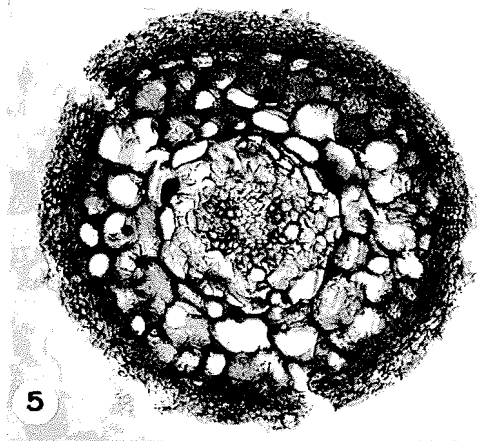
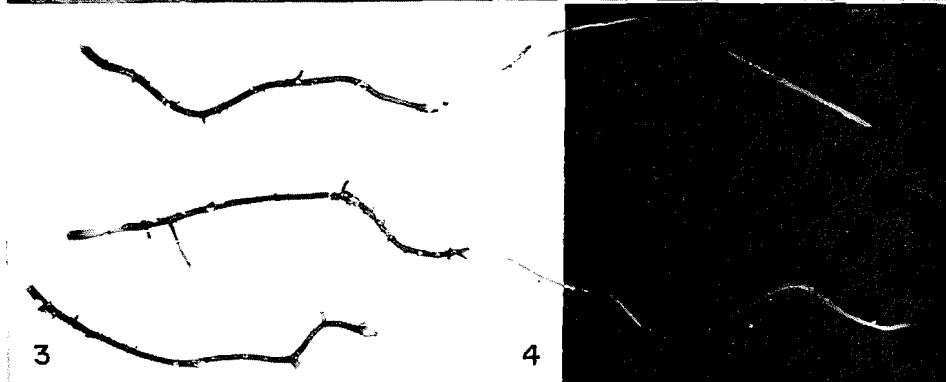
FIG. 2. *Pinus sylvestris* root culture showing mycorrhizae formed with *Amanita rubescens*: one of the mycorrhizae is dichotomous and rhizomorphs can be clearly seen (×10).

FIG. 3. *Pinus sylvestris* roots cultured on agar (×2). Note the swelling of the meristems (temperature 18 °C).

FIG. 4. *Pinus sylvestris* roots cultured in vermiculite (×2). Note the normal apices and the absence of laterals (temperature 22 °C).

FIG. 5. Transverse section of a mycorrhiza formed on an excised root culture showing the external mantle and Hartig's net (×40).

FIG. 6. Transverse section of a mycorrhiza formed on an excised root culture showing a longitudinal view of Hartig's net (×100).



mycorrhizae characteristically form) it was found that removal of the apical meristem had a favorable effect in this respect. Thereafter it was decided to prepare the explant as illustrated (Fig. 1c). The seedling was cut twice; first 5 mm above the junction between hypocotyl and root and then 35 mm lower on the root. The segment between these cuts was kept and will be referred to as an explant rather than isolated root since it contains a part of the root and a part of the hypocotyl.

The media for cultivating the roots were adapted from that designed by Slankis (1949), as follows; the trace elements solution was the same as above.

Organic medium	
Sucrose	175.0 g
Thiamine	1.0 mg
Choline chloride	2.5 "
Distilled water to	1000.0 ml
Mineral medium	
Ca(NO ₃) ₂ ·4H ₂ O	71.95 mg
MgSO ₄ ·7H ₂ O	20.47 "
KH ₂ PO ₄	10.00 "
Ferric citrate citric acid solution (1%)	1.00 ml
Trace elements solution	1.00 "
Distilled water to	1000.0 "

The organic components were solidified with 1.0% agar in 12 × 35 mm glass shell vials. The mineral medium was adjusted to pH 4.5 with diluted HCl and at first solidified with 1.0% agar in 11-cm Petri dishes. When vermiculite was used in place of the agar, 50 cm³ were moistened with 25 ml of the solution. When acid-washed quartz was used, 10 ml of the solution were poured over 50 g of the sand. The dishes were then wrapped individually in a paper towel and autoclaved. Thereafter, one explant was installed in each dish, the piece of hypocotyl being pushed into the agar of the vial; the root was partly buried in the vermiculite. Finally each Petri dish assembly was sealed with masking tape to prevent contamination and reduce evaporation. Incubation was in the dark at 21 ± 1 °C, unless otherwise stated, and its duration varied with the different experiments.

The inoculation of the explants was performed with a suspension of hyphae immediately after excision, and before the dishes were sealed. *Amanita rubescens* (Pers. ex Fr.) Quel. was used because its white color makes it conspicuous against vermiculite. The stock culture was transferred once monthly on solid medium containing the glucose and minerals of Melin and Rama Das (1954), with 1% malt. Hyphal suspensions were prepared as follows: a vigorous culture was first used to inoculate a 20-ml portion of the synthetic medium of Melin and Rama Das (1954), and after 10 to 11 days of incubation, the colony was homogenized in its own medium for 15 seconds in a semimicro Waring blender. Each root was inoculated with three drops of the suspension.

At the end of each experiment, growth was expressed as the difference between the final and the initial lengths of each root system, and the rate of growth was calculated on a weekly basis. The total number of root tips and

the total number of mycorrhizal tips being noted, it was then possible to calculate the percentage of rootlets which had formed mycorrhizae. The total number of dichotomous mycorrhizae was also noted for each treatment. Finally a few roots were fixed in formalin-acetic-alcohol and sectioned at 25–30 microns with a freezing microtome. The best sections were chosen under a binocular microscope, stained directly on a microscope slide placed first into 1% safranin for 15 seconds and then into 1% aniline blue in a saturated solution of picric acid for 20 seconds. The sections were thoroughly washed after each staining with distilled water. They were finally mounted and cleared in Shear's medium. The photographs were taken with a green filter.

Results and Discussion

All attempts to grow the explants on mineral agar were unsuccessful and resulted in an abnormal swelling of the meristems (Fig. 3), even if washed agar was used at different concentrations ranging from 0.6 to 1.5%. On the other hand, vermiculite gave a normal growth of the meristems (Fig. 4) either at 18 °C or at 25 °C. Quartz sand gave variable results; when the roots happened to grow at the surface, no swelling occurred, but when they grew into the substratum, as they usually do, the meristems did swell.

As mentioned previously, the presence of a piece of hypocotyl on the explant greatly accelerated the growth of the root. Table I shows the marked difference in growth between roots that were excised with or without a piece of their hypocotyl. When the hypocotyl was absent, growth stopped completely within 10 days.

TABLE I
Comparison of the growth of isolated roots and explants of the root hypocotyl of *Pinus sylvestris* (18 °C, 21 days)

Length of hypocotyl, mm	No. roots	Av. growth rate, mm/week	Av. no. laterals
0	18	1.6	0.0
10	23	9.6	0.5

This necessity of the hypocotyl for the successful growth of roots seems to be linked with Raggio's method. Indeed, Slankis (1947) could grow them by Robbin's technique without inclusion of a portion of the hypocotyl on the explants.

Since Slankis (1949) found a sharp optimum around 18 °C for the cultivation of roots of *P. sylvestris* by Robbins' technique, our first experiments, including those on the effect of the hypocotyl, were carried out at that temperature. Later, with the new technique, the optimum temperature was found to be around 22 °C, although growth was still active at either 18° or 25 °C (Table II).

The behavior of the explants of the root hypocotyl at different temperatures indicates that the growth obtained with Raggio's technique is more normal than the one obtained with Slankis' method. Indeed, the former allows growth to take place over a wide range of temperatures (18° to 25 °C at least) without showing any swelling of the meristems, while the latter gave an acute optimum

TABLE II
Comparison of the growth of explants of the root hypocotyl of *Pinus sylvestris* at different temperatures (19 days)

Temp., °C	No. roots	Av. growth rate, mm/week	Av. no. laterals
18	9	3.1	0.0
22	8	6.4	0.0
25	8	4.5	0.0

around 18 °C with swelling of the meristems (Slankis 1949). The roots of intact seedlings readily tolerate a temperature of 25 °C without swelling.

The effect of sucrose concentration in the vials was studied. A minimum of 7% sucrose in the organic medium was found necessary and good growth took place at concentrations ranging between 11 and 30%; thereafter 17.5% sucrose was used in the organic medium for the rest of the work.

A first attempt to induce the synthesis of mycorrhizae on roots on which the apical meristem was left intact failed to give any typical symbiotic roots; the roots were healthy but had very few short laterals. To promote the formation of the short roots, the germination period was extended from 14 to 28 days; as well, the apical meristem of each root was removed (Fig. 1c). By varying the length of the root tip removed, it was also possible to standardize the length of the segments at 35 mm. The results obtained from two experiments are summarized in Table III. A comparison of the uninoculated explants of this experiment with those of Tables I and II shows the effect of removing the root tip on the production of laterals. In Table III, it can also be seen that mycorrhizae formed on almost 100% of the segments and that 40% of the root tips were mycorrhizal.

TABLE III
Results of two experiments on the synthesis of mycorrhizae on explants of the root hypocotyl of *Pinus sylvestris*, 30 days after excision

	Uninoculated explants	Inoculated with <i>Amanita rubescens</i>	
		1st expt.	2nd expt.
No. segments used	9	20	15
No. segments with mycorrhizae	0	18	14
Average no. root tips per root	4.5	5.9	6.0
Average no. mycorrhizal root tips per root	0	2.5	2.4
% of mycorrhizal tips	0	42	40
Total no. dichotomic roots	0	5	0
Average rate of growth mm/week	4.0	3.5	5.3

When finishing the experiments on synthesis, we measured the pH of the solution in the vermiculite, and found that a shift had occurred in its value. The change was from an original pH 4.5 to a final pH 6.5. This is in agreement with the observation of Marx and Zak (1965) on the effect of vermiculite on the pH of a nutrient solution.

The mycorrhizae that were obtained were comparable in all respects with those forming on intact seedlings. Figure 2 illustrates three mycorrhizae, one of which is dichotomous; intensive formation of rhizomorphs occurred in the neighborhood of the mycorrhiza. Histological examination of swollen and fungus-covered laterals showed the outer sheath and the Hartig's net (Fig. 5). Critical examination revealed the occurrence of intercellular hyphae (Fig. 6).

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